Nanogels

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# Soluble Single-Molecule Nanogels of Controlled Structure as a Matrix for Efficient Artificial Enzymes**

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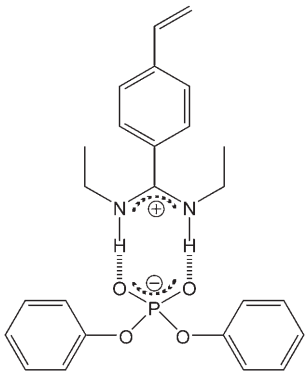
*Dedicated to Professor David C. Sherrington on the occasion of his 60th birthday*

For years, the construction of efficient artificial mimics of natural enzymes has been a challenging topic for scientists from a variety of disciplines.[1] Macrocyclic compounds, polymers, micelles, and others have been used as matrices for the construction of these mimics.[2] More recently, inspired by the concept of transition-state stabilization in enzyme catalysis[3] researchers have raised catalytically quite active antibodies against stable transition-state analogues of the reaction of interest.[4] Similarly, molecularly imprinted polymers[5] offer excellent possibilities for mimicking the active sites of enzymes since not only the shape of the transition state can be mimicked by imprinting but also suitable catalytic groups and binding sites can be introduced in a predetermined orientation.[6] However, only recently have compounds with strong catalytic activity clearly surpassing that of the corresponding catalytic antibodies been prepared.[7] The disadvantage of these molecularly imprinted model systems is their insolubility and the heterogeneity of the active sites.

Here we report on a new procedure to obtain molecularly imprinted catalysts in the form of soluble, single-molecule nanogels of defined structure. As they contain just one active site per particle, they are very similar to natural enzymes. More homogeneous imprinting has already been achieved by Zimmerman et al.,[8] who performed molecular imprinting inside dendrimers. But this method requires elaborate syntheses, and the selectivity in molecular recognition was modest. A more general method should be the synthesis of highly cross-linked, soluble nanogels. In this case it is crucial to avoid macrogelation during the polymerization and to produce separated, intramolecularly cross-linked macromolecules. Such polymers can be obtained, for example, by emulsion polymerization.[9] An elegant approach avoiding aqueous solutions during preparation has been described by Graham et al.[10] The polymerization is conducted in homogeneous solution at higher dilution in solvents with a solubility parameter $\delta$ similar to that of the corresponding polymer. Earlier work in our group[11] has shown that it is indeed possible to obtain molecularly imprinted, soluble, highly cross-linked nanogels by this method; however, the selectivity was rather low. Similar results were obtained by Resmini et al.;[12] the catalytic activity of their nanogels was also low with only 1 % of the prepared sites being catalytically active. They also did not control the size and the molecular weight of the nanoparticles. In general, nanogels of this type seem to be unsuitable for molecular imprinting because they possess a noncompact "fractal"[13] structure.

During the development of more efficient nanogels we used a rather simple catalytic system that was already employed with insoluble, molecularly imprinted polymers.[14] Imprinted nanogels were thus prepared by polymerization and cross-linking of complex **1**, which consists of a diphenyl



**1**

phosphate template as the stable transition-state analogue of the carbonate hydrolysis and *N*,*N*′-diethyl-4-vinylbenzamidine as the functional monomer. Removal of the template leads to active sites with a shape corresponding to the shape of the transition state and with one amidine group each. The ratio of the monomers and the content of cross-linker were the same as that in the preparation of insoluble macroporous polymers[14] (see Table 1). Radical polymerization was performed in dilute solution in cyclopentanone (0.1–1.5 wt %). (Cyclopentanone proved to be the best solvent for this purpose.) The resultant nanogels could be isolated from the completely transparent solution by precipitation with petroleum ether or by ultracentrifugation. The template was removed afterwards. Since the nanogels are completely soluble in THF, $CHCl_3$, DMF, $CH_2Cl_2$, DMSO, and in mixtures of water and acetonitrile, they could be investigated by typical analytical methods for polymers, such as gel permeation chromatography (GPC) and membrane osmometry, and kinetic experiments could be conducted in homogeneous solution.

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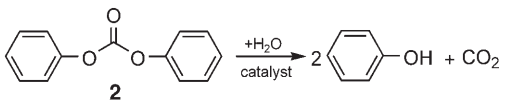
***Table 1:*** Preparation and molecular weights of imprinted nanogels **ING1**–**ING7**.[a]

| Nanogel | $c_{monomer}$ [%] | Method[b] | Yield [%] | $M_n$ ($\times 10^3$) | $M_w/M_n$ | $M_{n,abs.}$ ($\times 10^4$) | $M_{n,abs.}/M_n$ |
|---|---|---|---|---|---|---|---|
| **ING1** | 1.0 | standard | 53.6 | 23.1 | 8.5 | 38.1 | 16.5 |
| **ING2** | 1.0 | A | 81.3 | 29.5 | 10.4 | 49.2 | 16.7 |
| **ING3** | 1.0 | B, C | 83.7 | 24.5 | 6.0 | 62.4 | 25.5 |
| **ING4** | 1.5 | A, B, C, D | 99.5 | 42.2 | 18.6 | 92.4 | 21.9 |
| **ING5** | 0.5 | B, C | 61.1 | 8.91 | 3.6 | 26.1 | 29.3 |
| **ING6** | 0.1 | B, C | 27.6 | 1.34 | 1.54 | 4.43 | 33.0 |
| **ING7** | 0.1 | B, C, E | 31.4 | 1.30 | 1.54 | 3.90 | 30.1 |

[a] Standard conditions: The monomer mixture consists of 80 wt% of ethylene dimethacrylate, 11 wt% of methyl methacrylate, 9 wt% of **1**; addition of azobis(isobutyronitrile) (3 wt% of the monomer mixture). The mixture was heated in cyclopentanone at the stated concentration under nitrogen to 80 °C for 4 d. After evaporation to one-third of the volume, the product was isolated by precipitation by adding to of five times the volume of petroleum ether (b.p. 60–80 °C), ultrafiltration, and drying. The template was removed by dissolving the nanogel in chloroform and shaking the solution three times with aqueous 0.05 N NaOH solution. The organic layer was washed with neutral water, and the nanogel was isolated as before by precipitation. Apparent molecular weights ($M_n$, $M_w$) were determined in THF by GPC using linear polystyrenes as standards (for details see the Supporting Information). $M_{n,abs.}$ values were obtained by membrane osmometry in chloroform using regenerated cellulose membranes with pore sizes of 5 to 10 nm. [b] Variations: A) cross-linking content 90%. B) Stepwise polymerization at 60 °C (144 h), 70 °C (96 h), and 80 °C (96 h) with additional 1% initiator at 70 °C and 80 °C. C) In the "postdilution method" the monomer mixture was dissolved in the same volume of cyclopentanone and heated to 60 °C for 120 min. (This time was determined in advance from heating experiments until macrogelation occurred.) The mixture was then diluted with cyclopentanone to the desired monomer concentration (0.1–1.5%) and polymerized further in the stepwise temperature mode. D) Cross-linker trimethylolpropane trimethacrylate (TRIM). E) Template monomer **1** at half concentration (4.5%).

The preparation of the first nanogel (**ING1**, Table 1) was analogous to previous syntheses.[10,11] **ING1** has an apparent number-average molecular weight $M_n$ of 23100 (by GPC) and a high polydispersity of $M_w/M_n = 8.5$. The GPC analysis clearly shows that the nanogels are completely soluble. Membrane osmometry was employed to obtain information on the absolute number-average molecular weight ($M_{n,abs.}$) of the nanogel. It can be seen that $M_{n,abs.}$ is 16 times greater than $M_n$ (GPC). This is not surprising since highly cross-linked nanogels dissolved in good solvents possess a much more densely packed structure than the linear polystyrenes with the same molecular weight used as standards in GPC. The ratio $M_{n,abs.}/M_n$ therefore gives a good indication of the density of the nanogels.

The catalytic activity of the nanogels was determined by investigating the rate of hydrolysis of **2** [Eq. (1)] in HEPES buffer (pH 7.3)/acetonitrile (1:1). The kinetic data is given in Table 2. The kinetics were studied at low conversion, and pseudo-first-order rate constants ($k_{impr.}$) were calculated. The efficiency of the catalyst was expressed by the ratio of the rate constant in the presence of the nanogel catalyst to that in HEPES–AcCN solution ($k_{impr.}/k_{sol.}$). The ratio of the rate constants in the presence of the imprinted nanogel to that of a corresponding control nanogel ($k_{impr.}/k_{contr.}$) reflects effects connected to the imprinting in the nanogel (the imprinting effect[6a]).




**2** (1)

***Table 2:*** Catalytic activity of the imprinted nanogels **ING1**–**ING7**.

| Nanogel | $k_{impr.}$[a] ($\times 10^{-7}$) [$min^{-1}$] | $k_{impr.}/k_{sol.}$[b] | $k_{impr.}/k_{contr.}$[c] | Available cavities [$mmol\,g^{-1}$][d] | Cavities per particle |
|---|---|---|---|---|---|
| **ING1** | 3.59 | 14.8 | 1.42 | 0.067 | 25.5 |
| **ING2** | 6.16 | 25.3 | – | 0.097 | 47.7 |
| **ING3** | 12.8 | 52.7 | 5.52 | 0.073 | 45.7 |
| **ING4** | 70.9 | 291.4 | 18.5 | 0.102 | 94.5 |
| **ING5** | 3.77 | 15.5 | 3.20 | 0.070 | 18.2 |
| **ING6** | 3.48 | 14.3 | 2.58 | 0.041 | 1.81 |
| **ING7** | 3.91 | 16.1 | 2.43 | 0.027 | 1.03 |

[a] Hydrolysis of **2** in a solution of 50 mM HEPES buffer (pH 7.3)/MeCN (1:1) at 10 °C with 2 equiv of cavities to 1 equiv of substrate. (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid). $k_{impr.}$: pseudo-first-order rate constant in presence of the imprinted nanogel. [b] $k_{sol.}$: rate constant in HEPES buffer (pH 7.3)/MeCN (1:1). [c] $k_{contr.}$: rate constant in presence of the control nanogel. [d] Available cavities with amidinium groups determined by potentiometric titration in aqueous 0.1 N NaCl/MeCN (1:1) solution with 0.02 N aqueous HCl.

Control nanogels were prepared in analogy to the imprinted ones (**ING1**–**ING7**), but instead of the template diphenyl phosphate, formic acid was used (**CNG1**–**CNG7**; see the Supporting Information). The standard nanogel **ING1** shows clear catalytic activity, but it is lower than that of the corresponding insoluble macroporous polymers.[14] To obtain more rigid nanogels with better stabilization of the active site by more efficient cross-linking, we employed new methods of preparation (see the Supporting Information); some representative data is shown in Tables 1 and 2.

Higher monomer concentrations and higher degrees of cross-linking resulted in higher molecular weights and considerably higher polydispersities $M_w/M_n$, but at the same time the catalytic activity of the nanogels also increased. The potential for increasing the monomer concentration is limited because at concentrations above the critical concentration $c_m$ (in the present case at around 1.5 wt%) macrogelation takes place. A further considerable improvement of the properties was achieved when the polymerization was performed in three stages with increasing temperatures (60, 70, and 80 °C). The most effective increase in the rigidity of the nanogel structure was obtained by a novel method we have called the "postdilution method". It is based on the observation that the

in the homopolymerization at high EDMA concentration the cross-linking degree of the system reaches its maximum at a point just before macrogelation takes place.[15] To avoid macrogelation we stopped the polymerization at high concentration just prior to macrogelation and then diluted extensively with cyclopentanone to keep the concentration of the polymerization solution below $c_m$ (0.1–1.5 wt.%). In this way further polymerization results in an increase of the ratio $M_{n,abs.}/M_n$, a measure of the density of the particles, from around 16 to values of 26–33 with EDMA as cross-linker (see Table 1). At the same time the polydispersity became lower, and most importantly, the catalytic activity was greatly improved.

Since the trifunctional cross-linker trimethylolpropanetrimethacrylate (TRIM) had a positive influence on the catalytic properties of the nanogels, it was used in the preparation of **ING4**. Here, all possibilities for improvement were combined, and a very efficient catalyst system was obtained with the highest $k_{impr.}/k_{sol}$ value of 291 and a $k_{impr.}/k_{contr.}$ value of 18.5. Like natural enzymes, **ING4** shows typical Michaelis–Menten kinetics for the catalysis of carbonate hydrolysis. A plot of initial reaction rates versus the substrate concentration shows saturation kinetics (see the Supporting Information). From this data the Michaelis constant $K_m = 1.82$ mM and the turnover number $k_{cat} = 7.27 \times 10^{-5}$ min$^{-1}$ can be calculated. The $k_{cat.}/k_{uncat.}$ ratio reached the remarkable value of 2990. A control polymer (**CNG4**) showed considerably lower catalytic activity, and no saturation phenomena were observed (nearly a straight line in the plot in Figure SI-2).

These results clearly indicate that the catalysis is occurring inside the active site. Thus, it was possible for the first time to prepare a soluble imprinted nanogel with catalytic activity similar to that of insoluble imprinted polymers. A further improvement due to the solubility of the catalysts was not observed. In the preparation of the nanogels the yields and the number of obtained active sites were nearly quantitative. This procedure, therefore, should also be suitable for more sophisticated and efficient catalytic systems with rate enhancements of more than 100000 fold, as has been described previously.[7] In addition it should also be applicable to the frequently used noncovalent imprinting protocols based on weaker interactions[5b] since during the imprinting no water or other protic solvents are present.

After obtaining nanogels with high rigidity and good catalytic activity, we tried again to reduce the heterogeneity by controlling the molecular weight and the polydispersity of the catalysts. This could be achieved by using higher dilution during the preparation in the final step of the "postdilution method". **ING3**, **ING5**, and **ING6** were prepared under identical conditions, except the dilution was 1.0, 0.5, and 0.1%. As can be seen in Table 1 the $M_{n,abs.}$ dropped from 624 to 261 and to 44.3 kDalton. At the same time, the polydispersity $M_w/M_n$ dropped from 6.0 to 3.6 and 1.54. The reason is a much lower aggregation of the primary particles. A polydispersity of 1.54 is an extremely good value for a radical polymerization. A calculation based on the experimental results for **ING6** show the existence of on average 1.8 active sites per particle. As expected, particles of higher molecular weight contain, on average, a higher number of active sites per particle. To obtain nanogels with an average of one active site per particle, we reduced the amount of template monomer **1** in the preparation of **ING7**. Thus we obtained for the first time soluble nanogels with molecular weights similar to those of natural enzymes, around 40 kDalton, very low polydispersity, and on average only one active site per particle.

We visualized and characterized these nanogels by scanning transmission electron microscopy (STEM).[16] **ING6** was prepared without drying the material in order to avoid any additional aggregation and was subsequently analyzed. $RuO_4$-stained particles of **ING6** in dilute chloroform solution were applied to a STEM copper grid covered by fenestrated carbon film. The STEM overview picture in Figure 1a shows a narrow size distribution, and at higher magnification (Figure 1b) spherical particles with diameters between 10 and 20 nm can be clearly seen. These particles represent single, intramolecularly cross-linked macromolecules that do not possess a marked fractal structure. The particles are rigid and do not collapse onto the support film. With the available data still no safe conclusions about the fine structure of the nanogels can be obtained from these images.

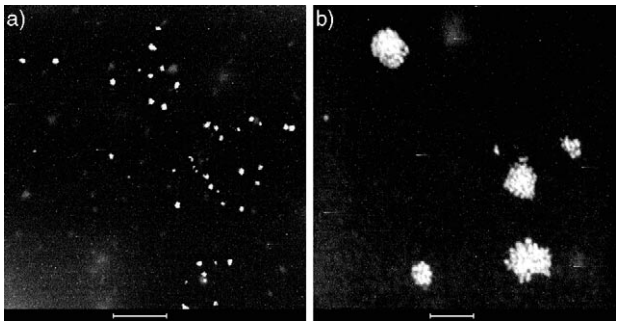



***Figure 1.*** STEM pictures of **ING6**. a) Overview: $RuO_4$-stained nanoparticles (scale bar: 200 nm). The size distribution is small. b) At higher magnification the structure of the spherical particles can be seen (scale bar: 20 nm). The diameter of particles of 10–20 nm can be distinguished. The investigation was performed with a Philips Tecnai F30 analytical TEM instrument in STEM mode through a "high-angular annular dark field" detector (HAADF). The presence of Ru was confirmed through energy-dispersive X-ray spectroscopy with an Oxford Pentafet (Si/Li) EDX detector. Ru is homogeneously distributed on the nanogel and forms nanosized clusters on the surface of the polymer. Through a tilt series in the measurement of approximately $\pm 40°$ the shape and the size of the particles did not change.

Since these soluble particles of 40 kDalton can be prepared, on average, with one active catalytic site per particle and show Michaelis–Menten kinetics, a high analogy to natural enzymes was achieved. These catalysts can be analyzed and handled like enzymes but they are by far more stable. As with enzymes, it should also be possible to purify and enrich these nanogels by enzyme methodology, for example, by affinity chromatography. In addition to their importance as enzyme mimics, these novel soluble nanogels combining low molecular weight and low polydispersty with

densely packed structures might find widespread use in different areas of science and technology.

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